

## Inhibitory effect of corticosteroids on the secretion of tumour necrosis factor (TNF) by monocytes is dependent on the stimulus inducing TNF synthesis

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### SUMMARY

The cytokine tumour necrosis factor (TNF) is believed to be involved in the pathophysiology of several human disease states, both septic and non-septic. Different pathways of induction are involved in the generation of TNF in these disease states. We therefore used four different stimulatory agents, lipopolysaccharide, phorbol myristate acetate, silica quartz, and anti-human IgG antibody to study the influence of the corticosteroids prednisolone and budesonide on the secretion of TNF by human monocytes. Both prednisolone and budesonide inhibited TNF secretion induced by these four stimulating agents in a different degree. Inhibition was strong when TNF secretion was induced by lipopolysaccharide or anti-human IgG antibody. A weaker inhibitory effect was observed when TNF secretion was induced by silica quartz. Only minimal inhibition of phorbol myristate acetate induced TNF secretion was observed. Furthermore, it is shown that inhibition is dependent on the dose of corticosteroid, but not or only minimally on the dose of stimulating agent, indicating that inhibition cannot be overcome by increasing the cell-activating stimulus. Finally, optimal inhibition of TNF secretion by corticosteroids is shown to be dependent on the presence of corticosteroids during the phase of cell stimulation.

**Keywords** tumour necrosis factor inhibition corticosteroids

### INTRODUCTION

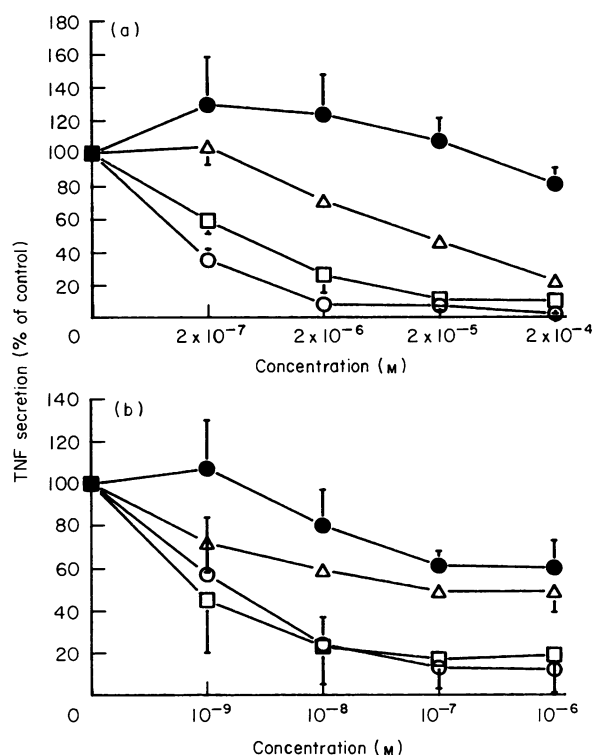
Tumour necrosis factor (TNF) is an inflammatory and immunoregulatory cytokine produced mainly by monocytes and macrophages after stimulation with endotoxin (Beutler *et al.*, 1986; Kornbluth & Edgington, 1986). Other cells of the immune system, like T lymphocytes (Cuturi *et al.*, 1987) and natural killer cells (Christmas, Meager & Moore, 1987) have also been shown to produce TNF after adequate stimulation. TNF has numerous regulatory functions in immune processes, thus playing a pivotal role in the enhancement of inflammation and immunity (Beutler & Cerami, 1987). Besides its physiological role TNF is also believed to be involved in the pathophysiology of a number of disease processes like sepsis (Tracey *et al.*, 1988; Waage, Halstensen & Espevik, 1987), cachexia or anorexia accompanying cancer or chronic infection (Cerami *et al.*, 1985; Oliff *et al.*, 1987; Tracey *et al.*, 1988), parasitic infections (Scuderi *et al.*, 1986; Clark *et al.*, 1987), allograft rejection (Maury & Teppo, 1987), graft-versus-host disease (Piguet *et al.*,

1987), and autoimmune diseases like rheumatoid arthritis (Saklatvala, 1986; Bertolini *et al.*, 1986; Thomson, Mundy & Chambers, 1987) and systemic lupus erythematosus (SLE) (Jacob & McDevitt, 1988).

The corticosteroid drugs prednisolone and budesonide have both been shown to inhibit inflammatory processes and to suppress the immune response in several autoimmune diseases or during allograft rejection (Ruers *et al.*, 1987; 1988). One important mechanism underlying the anti-inflammatory and immunosuppressive effects of corticosteroids is the inhibition of production and secretion of inflammatory mediators by immunocompetent cells (Cupps & Fauci, 1982; Ruers *et al.*, 1987). For example, corticosteroids have been shown to inhibit production of interleukin-1 (IL-1) and IL-2, and of interferon- $\gamma$  (Arya, Wong-Staal & Gallo, 1984; Knudsen, Dinarello & Strom, 1987; Kern *et al.*, 1988).

Corticosteroids have also been shown to inhibit TNF production both by murine and rat macrophages (Beutler *et al.*, 1986; Waage, 1987) and by human monocytes (Waage & Bakke, 1988). In these studies endotoxin was used to stimulate TNF production. Besides endotoxin, other compounds have been shown to induce TNF production. The protein kinase C-

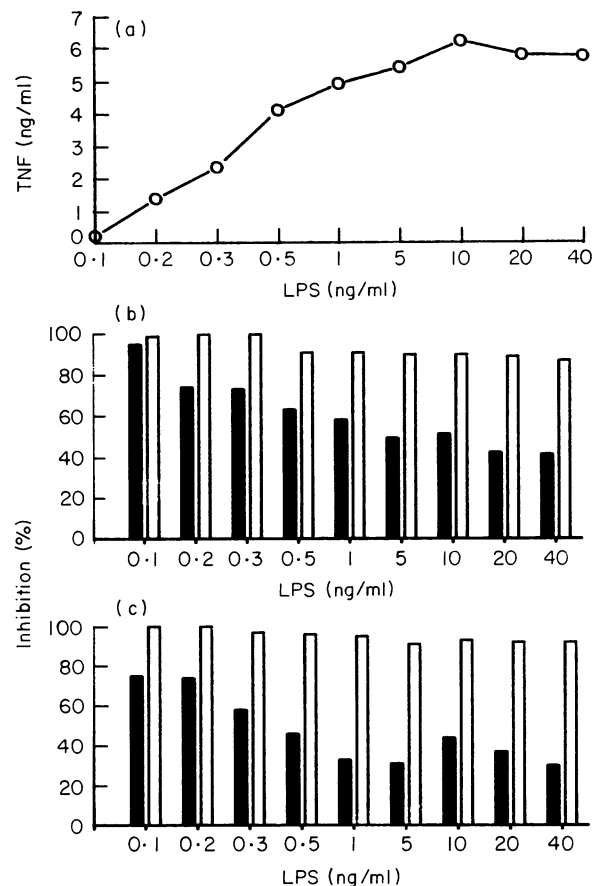
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**Fig. 1.** Inhibition by corticosteroids of TNF secretion induced by four different stimulating agents. Monocytes were incubated with various concentrations of either prednisolone (a) or budesonide (b). After 4 h, the stimulating agents were added to the cultures. After 17 h, samples of supernatant were harvested to measure TNF concentration. Data represent mean  $\pm$  s.e.m. of triplicate determinations of four different individuals. TNF secretion is expressed as percentage of TNF secretion in the absence of corticosteroids. O, anti-human kapa ( $2.2 \mu\text{g/ml}$ ); ●, phorbol myristate acetate (10 ng/ml);  $\Delta$ , silica (0.5 mg/ml); and  $\square$ , lipopolysaccharide (10 ng/ml).

activating agent phorbol myristate acetate (PMA) is a potent inducer of TNF production by monocytes (Nedwin *et al.*, 1985). Recently we have demonstrated that TNF secretion by monocytes can be induced through cross-linking of monocyte Fc receptors (FcR), for example with an anti-human IgG antibody or with immune complexes (Debets *et al.*, 1988a). Furthermore, data from our laboratory demonstrate that silica quartz, a known inducer of IL-1 production in monocytes (Lepe-Zuniga & Gery, 1984), can also induce TNF production by monocytes (Borm *et al.*, 1988). The pathways underlying the induction of TNF synthesis by these different compounds are likely to be involved in the generation of TNF in non-septic disease states like allograft rejection, autoimmune diseases like rheumatoid arthritis, and pneumoconiosis (Borm *et al.*, 1988). We therefore investigated the influence of corticosteroids on the secretion of TNF by monocytes after stimulation with these compounds.

It is shown that TNF secretion is variably inhibited by corticosteroids, depending on the type of stimulus used. The strongest inhibition is found when TNF secretion is induced with lipopolysaccharide (LPS) or anti-human IgG antibody, inhibition of PMA- and silica-induced TNF secretion being considerably weaker.



**Fig. 2.** Influence of the concentration of lipopolysaccharide (LPS) on inhibition of TNF secretion by corticosteroids. Monocytes were preincubated with budesonide (b) or prednisolone (c) at concentrations giving optimal (open bars) or suboptimal (closed bars) inhibition of LPS-induced TNF secretion, as determined by the former experiments. Optimal inhibitory concentrations chosen were  $10^{-7}$  and  $2 \times 10^{-5}$  M for budesonide and prednisolone, respectively. Suboptimal inhibitory concentrations chosen were  $10^{-9}$  and  $2 \times 10^{-7}$  M for budesonide and prednisolone, respectively. After 4 h, LPS at different concentrations was added to the cultures. After 17 h, samples of supernatant were harvested to measure TNF concentration. Values in (b) and (c) are means of triplicate determinations of two different individuals. Data are expressed as percentage inhibition of TNF secretion, which is defined as  $(1 - \text{TNF [with corticosteroids]} / \text{TNF [without corticosteroids]}) \times 100$ . (a) Actual dose-response curves of LPS-induced TNF secretion in these two individuals (expressed as mean of triplicate determinations of both individuals).

## MATERIALS AND METHODS

### Materials

Prednisolone was purchased from Centrachemie (Etten-Leur, The Netherlands). A stock solution was prepared of 25 mg/ml prednisolone in water. Budesonide was kindly provided by Dr R. Brattsand (Drako/Astra, Lund, Sweden). A stock solution of  $10^{-2}$  M budesonide was prepared in 96% ethanol. Both stock solutions were further diluted in culture medium to obtain the final concentrations used in the experiments. Monocytes cultured in medium containing equivalent amounts of ethanol without budesonide were used as controls for budesonide-treated cells. Monocytes cultured in medium without any

additions were used as controls for the prednisolone-treated cells. LPS (from *Escherichia coli* 055:B5) was purchased from Difco (Detroit, MI). PMA and silica quartz (particle size 0.007  $\mu\text{m}$ ) were purchased from Sigma (St Louis, MO). Goat F(ab')<sub>2</sub> anti-human kappa chain antibody was purchased from Tago (Burlingame, CA).

### Cells

Buffy coats obtained after cytophoresis of cells from healthy donors were kindly provided by the local blood bank. Monocytes were isolated as described elsewhere (Graziano & Fanger, 1987). Briefly, mononuclear cell suspensions obtained after Ficoll-Isopaque centrifugation were allowed to clump by low-speed centrifugation at 4°C. Cell clumps consisting for more than 90–95% of monocytes as evidenced by esterase staining were separated from the rest of the cells by sedimentation through ice-cold bovine calf serum (BCS; Hyclone, Logan, UT). Monocytes were cultured in 96-well, flat-bottomed culture plates (Costar, Cambridge, MA) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% heat-inactivated BCS, 100 IU/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin (Flow, Irvine, UK). In each well  $2.5 \times 10^4$  monocytes were cultured in 300  $\mu\text{l}$  culture medium. After indicated time periods 125- $\mu\text{l}$  samples of supernatant, from which cells were removed by centrifugation of the plates for 5 min at 1200 rpm, were harvested and kept frozen at  $-20^\circ\text{C}$  for measurement of TNF concentration at a later date. Viability of cells was tested by trypan blue exclusion and exceeded 95% in all experiments. All experiments were performed in duplicate or triplicate.

### TNF-ELISA

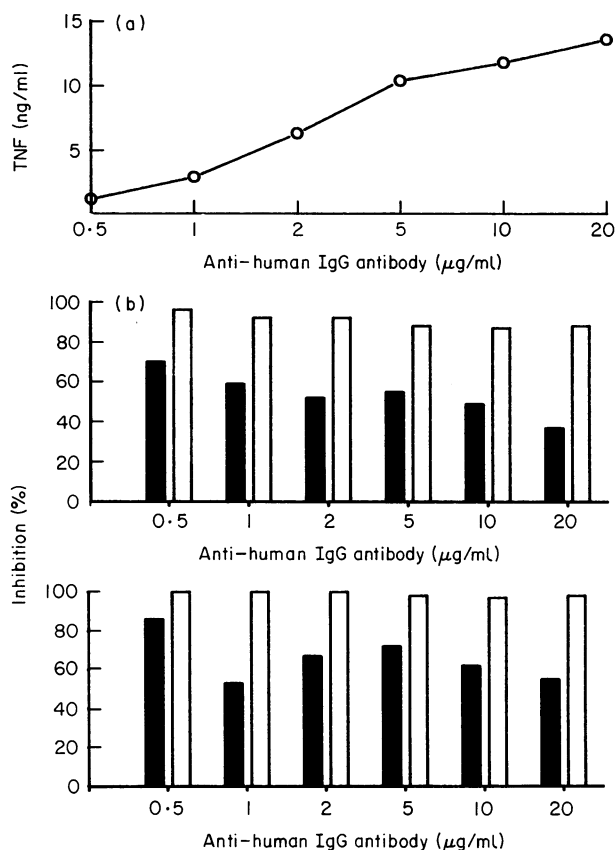
TNF concentration of the supernatants was determined with a TNF-specific ELISA (Debets *et al.*, 1988b), with slight modifications as described before (Debets *et al.*, 1988a).

### Statistical analysis

Student's *t*-test for paired and unpaired data was used for statistical analysis of the data.

## RESULTS

LPS, PMA, silica and anti-human IgG antibody were used as stimulating agents to study the influence of corticosteroids on TNF secretion by monocytes. Concentrations of each of the stimulating agents giving optimal stimulation of TNF secretion were determined in separate experiments (data not shown). These concentrations were used in the first set of experiments, in which the effect of a 4-h pre-incubation period with various concentrations of the corticosteroids prednisolone and budesonide on TNF secretion by monocytes was studied. TNF secretion was determined by measuring TNF concentration of the cell-free supernatant of monocyte cultures incubated for 17 h with the different stimulating agents in the presence or absence of corticosteroids. TNF secretion in the absence of corticosteroids was 2.9, 2.2, 5.4, and 0.77 ng/ml, when using LPS, anti-human IgG antibody, PMA or silica, respectively, as stimulating agents (mean of triplicate determinations of four different individuals). Both prednisolone and budesonide inhibit TNF secretion in a dose-dependent manner. The strongest inhibition was seen when TNF secretion was induced with LPS and anti-



**Fig. 3.** Influence of the concentration of anti-human IgG antibody on inhibition of TNF secretion by corticosteroids. See legend for Fig. 2 for a, b, and c and for calculation of inhibition. Here the stimulating agent is the antibody instead of LPS.

**Table 1.** Effect of pre-incubation of monocytes with corticosteroids on lipopolysaccharide (LPS) induced TNF secretion

Pre-incubation with	LPS added (ng/ml)	TNF (pg/ml)
—	100	4458 ± 2422
Prednisolone	100	5564 ± 3162*
—	100	4085 ± 1700
Budesonide	100	2673 ± 1491†

Mean ± s.e.m. of nine individuals, each determined in triplicate.

\*Not significant *versus* control.

†*P* < 0.05 *versus* control.

human IgG antibody. PMA-induced TNF secretion was only moderately inhibited, especially with prednisolone, which even enhanced TNF secretion at lower concentrations. Silica-induced TNF secretion was also inhibited by both corticosteroids, the degree of inhibition being less than that observed after stimulation of TNF secretion with LPS and anti-human IgG (Fig. 1).

We then examined whether the degree of inhibition of TNF secretion by corticosteroids was dependent on the dose of stimulating agent added to the monocytes. As an example of the

**Table 2.** Partial reversibility of inhibitory effect of budesonide

Pre-incubation with	Stimulation phase	Inhibition (%)
Budesonide	Budesonide	80 ± 14
Budesonide	—	25 ± 11*

Percentage inhibition of TNF secretion was calculated as:

$$1 - \frac{\text{TNF (budesonide)}}{\text{TNF (control)}} \times 100$$

where TNF (budesonide) represents TNF secretion by monocytes which are either only pre-incubated with budesonide or cultured in the continual presence of budesonide, and TNF (control) represents TNF secretion by monocytes which are cultured in the absence of budesonide. Data represent mean ± s.e.m. of triplicate determinations of four different individuals.

$P < 0.01$ .

dose-response relationship of LPS-induced TNF secretion, data from two individuals are given in Fig. 2. Two different concentrations of the corticosteroids prednisolone and budesonide were used to inhibit TNF secretion, one giving optimal inhibition of TNF secretion and one giving suboptimal inhibition of TNF secretion, as determined by the experiments shown in Fig. 1. At optimal inhibitory concentrations of prednisolone or budesonide inhibition of TNF secretion was not affected by the dose of LPS added to the monocyte cultures. However, at suboptimal inhibitory concentrations of both corticosteroids a distinct reduction in inhibition was observed when the amount of LPS added to the cultures was increased (Fig. 2b, c). The same was essentially found when TNF secretion was induced through cross-linking of monocyte FcR with anti-human IgG antibody. The influence of the amount of anti-kappa on the degree of inhibition by corticosteroids was, however, less than that observed with LPS (Fig. 3).

Next we examined whether the inhibition of TNF secretion by corticosteroids requires the presence of corticosteroids at the time of addition of stimulating agents to the monocytes. Monocytes were pre-incubated for 4 h with prednisolone or budesonide at optimal inhibitory concentrations, after which the corticosteroids were washed away. After 4 h, monocytes were stimulated with LPS. As can be seen in Table 1, monocyte TNF secretion was not inhibited by a 4-h pre-incubation period with prednisolone. TNF secretion was even enhanced by pre-incubation with prednisolone, although the difference did not reach statistical significance. After a 4-h pre-incubation period with budesonide, TNF secretion was still significantly suppressed (Table 1). However, the degree of inhibition of TNF secretion was significantly less than that observed in the continual presence of budesonide, indicating that the inhibition of TNF secretion is partially reversed after removal of budesonide from the cultures (Table 2).

## DISCUSSION

We have shown that corticosteroids inhibit the secretion of TNF by human monocytes in a dose-dependent manner. Both prednisolone, which is used as a systemic immunosuppressive drug in clinical medicine, and budesonide, which is used mainly as a topical anti-inflammatory drug in respiratory diseases, were shown to inhibit TNF secretion. Budesonide proved to be a

more potent inhibitor of TNF secretion than prednisolone (approximately 100 times more potent on a molar basis). Corticosteroids variably inhibited TNF secretion, depending on the kind of stimulus used to induce TNF secretion. The strongest inhibition was observed when TNF secretion was induced by a 'receptor-mediated' mechanism, i.e. with LPS and through cross-linking of monocyte FcR with an anti-human IgG antibody. The inhibition was much less or, at lower concentrations of corticosteroids, even totally absent, when TNF secretion was induced by the protein kinase C-activating agent PMA. The inhibition observed when TNF secretion was induced by silica, which non-specifically activates monocytes, was intermediate in comparison with PMA-induced and LPS- or anti-human IgG antibody-induced TNF secretion. The degree of inhibition is not merely dependent on the stimulus strength of the variable agents inducing TNF secretion. Although inhibition of TNF secretion induced by PMA, which is the most potent stimulus, is very weak, TNF secretion induced by LPS or anti-human IgG antibody, which also strongly stimulate TNF secretion, is almost completely inhibited by corticosteroids. Furthermore, TNF secretion induced by silica, which is the least potent of the stimuli used, is inhibited considerably less than LPS- or anti-human IgG antibody-induced TNF secretion. These data suggest that, as far as cytokine secretion is concerned, corticosteroids interfere more potently with receptor-transmitted cellular activating pathways than with protein kinase C-mediated cellular activation.

The results shown in Figs 2 and 3 indicate that the inhibition by corticosteroids of TNF secretion induced by LPS and anti-human IgG antibody is not of a competitive nature. The inhibition by optimal inhibitory concentrations of corticosteroids cannot be eliminated by increasing the dose of stimulatory agents. Only at suboptimal inhibitory concentrations of corticosteroids was a slight reduction of inhibition observed when the concentration of LPS or anti-human IgG antibody was increased.

Prednisolone-induced inhibition was shown to be rapidly reversible. Four hours after removal of prednisolone from the culture medium, inhibition of TNF secretion was no longer observed. Budesonide-induced inhibition was less rapidly reversed, significant inhibition still being present four hours after removal of budesonide from the cultures. Since the washing procedure was identical for both corticosteroids, it seems unlikely that the inhibition observed after pre-incubation with budesonide is due to incomplete removal of the drug from the culture medium, since this would have resulted in persistent inhibition in the prednisolone-pretreated cultures as well. The observed difference in duration of inhibition suggests that budesonide is less rapidly cleared from the intracellular compartment, possibly because of greater affinity for intracellular steroid-binding receptor molecules. This is also in accordance with the fact that budesonide is, on a molar basis, a far more potent inhibitor of TNF secretion than is prednisolone.

Endotoxin-induced TNF production has been shown to be inhibited by corticosteroids in previous studies, both in murine and in human systems (Beutler *et al.*, 1986; Waage & Bakke, 1988; Waage, 1987). Dexamethasone was shown to inhibit endotoxin-induced mouse macrophage TNF production at transcriptional as well as post-transcriptional levels (Beutler *et al.*, 1986). Only short periods of exposure to dexamethasone were necessary to fully block TNF production, and inhibition

was present even when dexamethasone was administered to the cells at the same time as endotoxin. Human monocyte TNF secretion induced by endotoxin was also shown to be inhibited by dexamethasone, but required a much longer period (48 h) of pre-incubation of cells with dexamethasone for inhibition to become optimal (Waage & Bakke, 1988). Our study shows that corticosteroids also inhibit monocyte TNF secretion induced by stimuli other than endotoxin. In addition, we found that only short periods of exposure (4 h) to prednisolone or budesonide were needed to inhibit TNF secretion optimally.

TNF is believed to be one of the prime mediators of sepsis on the basis of both experimental animal studies (Tracey *et al.*, 1986; 1988) and on clinical studies in humans (Waage *et al.*, 1987; Girardin *et al.*, 1988; Debets *et al.*, 1989). Numerous animal studies have demonstrated a protective effect of corticosteroids against mortality in experimental sepsis (reviewed by Hinshaw *et al.*, 1987). However, a beneficial effect of corticosteroids in human sepsis has not yet been convincingly demonstrated (Sprung *et al.*, 1984; Hinshaw *et al.*, 1987; Bone *et al.*, 1987). The strong inhibitory effect of corticosteroids on endotoxin-induced TNF secretion may be one of the mechanisms underlying the protective effect of corticosteroids in experimental animal sepsis.

We have recently demonstrated that TNF secretion by monocytes can be induced through cross-linking of the FcR, for example with immune complexes or with an anti-human IgG antibody (Debets *et al.*, 1988a). This finding suggested that TNF may play a role in the pathophysiology of immune complex diseases, for example rheumatoid arthritis. Several investigations support this hypothesis. TNF has been shown to induce bone and cartilage resorption *in vitro*, partly in synergism with IL-1 (Saklatvala, 1986; Thomson *et al.*, 1987; Stashenko *et al.*, 1987). Furthermore, TNF has recently been detected in synovial fluid of patients with rheumatoid arthritis (Saxne *et al.*, 1988). These disease states can be potentially suppressed by corticosteroids. Our finding that corticosteroids inhibit TNF secretion induced by FcR cross-linking helps to explain the mechanisms underlying the beneficial effect of corticosteroids in these disease states.

TNF shares many of its biological activities with another important immunoregulatory cytokine, IL-1. Corticosteroids have also been shown to inhibit IL-1 synthesis by human monocytes (Kern *et al.*, 1988) and by a human monocyte-like cell line (Knudsen *et al.*, 1987). IL-1 synthesis by monocytes is mainly inhibited at post-transcriptional level (Kern *et al.*, 1988). The exact cellular level at which corticosteroids interfere with human monocyte TNF production and secretion remains subject of further study.

In conclusion, this study shows that the corticosteroids prednisolone and budesonide variably inhibit TNF secretion by monocytes, depending on the stimulus used to induce TNF secretion. Strong inhibition was found when TNF secretion was induced by LPS or by FcR cross-linking, weak inhibition was found with PMA-induced TNF secretion, and inhibition was intermediate with silica-induced TNF secretion. These differences may be of importance in explaining the variable effects of corticosteroids in several human disease states.

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